UNIVERSITI PUTRA MALAYSIA

THE EXPRESSION OF CHICKEN ANEMIA VIRUS VP3 GENE AND INDUCTION OF APOPTOSIS IN TRANSFORMED AND TUMOR CELLS

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THE EXPRESSION OF CHICKEN ANEMIA VIRUS VP3 GENE AND INDUCTION OF APOPTOSIS IN TRANSFORMED AND TUMOR CELLS

By

MOHAMED GHRICI

Thesis Submitted in Fulfilment of the Requirement for the
Degree of Master of Science in the Faculty of Veterinary Medicine
Universiti Putra Malaysia

October 2001

To my father GHRICI MOHAMED and my mother KHIDER YAMINA

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Master of Science.

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Chairman : Associate Professor Dr Mohd Azmi Mohd Lila, Ph.D.

Faculty : Veterinary Medicine

The pathogenicity of chicken anemia virus (CAV), as shown in previous studies, is the function of apoptotic mechanism as observed in chicken thymocytes and transformed chicken-lymphoblastoid T cells. Thus the present study aimed to investigate the gene and its gene product responsible for apoptosis in as such that leads to the destruction of affected cells. It is known that apoptosis process is an important natural physiological mechanism that induces killing of cancer cells. Therefore, in theory, the gene or its gene product that is responsible for the apoptotic mechanism could be exploited especially in cancer therapy. In this study, a complete open reading frame (ORF) encoding VP3 protein was obtained from the DNA extracted from archival paraffin-embedded tissues and cloned into a plasmid vector. The sequencing of the full length of the ORF encoding VP3 protein showed that it was 363 base pairs long, which is similar in size to that of the reference CAV Cux-1 strain. Comparison of the nucleotide sequences of this VP3 gene with that of the CAV Cux-1 strain exhibited 98% sequence homology indicating that these two viruses are closely related. The functional characteristics of VP3 gene were further investigated by using an eukaryotic expression system, plasmid pcDNA 3.1/Zeo+.

The purified recombinant pcDNA-VP3 plasmid was used to transfect mammalian cells and cancer cells via electroporation. The induction of apoptosis, upon expression of VP3 gene, was studied by identifying and detecting morphological and biochemical changes due to apoptosis. Upon electron microscopic examination, typical apoptotic features were observed, which includes nuclear margination and formation of apoptotic bodies, as early as two days after transfection. Morphological changes developed due to apoptosis were obvious which were later confirmed by means of the apoptotic biochemical staining and DNA fragmentation assay. The differential activities of VP3 were further investigated by the use of the immunofluorescence technique. The VP3 protein was expressed and detected only in the cytoplasm of normal cells. In contrast, the expression of VP3 protein was localized particularly in the nucleus of the transformed cell lines (Vero and rat embryo fibroblast cells) and human derived breast cancer cells (MCF-7 and MDA-MB 231). This differential cellular localization of VP3 protein in normal versus tumorigenic and transformed cells was the reason of VP3 specifically inducing apoptosis in transformed and cancerous cells but not in normal cells. The effects of VP3 protein expression in these three types of cells were also confirmed upon examination by confocal microscopy analysis with cells stained in propidium iodide and acridine orange stains. In conclusion, VP3 protein expression alone was able to induce apoptosis in transformed cells and in human derived cancer cells but had no effect on normal cells. The substantial evidences indicated that the DNA construct containing the VP3 gene under the control of human cytomegalovirus (hCMV) promoter, alias in the form of a DNA vaccine is the new potential candidate for anti-cancer therapy.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia
sebagai memenuhi keperluan ijazah Master Sains

EKSPRESI GEN VP3 VIRUS ANEMIA AYAM YANG MENGARUH APOPTOSIS DALAM SEL-SEL YANG TERUBAH DAN SEL-SEL KANSER

Oleh

MOHAMED GHRICI

Oktober 2001

Pengerusi: **Profesor Madya Mohd Azmi Mohd Lila, Ph.D.**

Fakulti: **Perubatan Veterinar**

Sebagaimana yang telah ditunjukkan pada kajian terdahulu, permulaan pegembangan penyakit Virus Anemia Ayam (CAV) yang mempunyai fungsi mekanisma apoptotik yang terdapat dalam timosit-timosit ayam dan limphoblastoid sel-sel T ayam yang terubah. Kajian ini bermatlamat untuk menyiasat gen dan hasil keluaran gen yang bertanggung-jawab terhadap apoptosis seperti meningkatnya kerosakkan kepada sel-sel yang terjangkit. Sebagaimana yang diketahui, proses apoptosis adalah mekanisma fisiologikal semulajadi yang penting dalam mengaruh pembunuhan sel-sel kanser. Oleh itu secara teori, gen atau hasilan gen yang bertindakbalas bagi mekanisma apoptosis boleh dipergunakan terutamanya dalam pengubatan kanser. Dalam kajian ini, rangka bacaan terbuka yang lengkap (ORF) yang mengekod protin VP3 telah diperolehi daripada DNA yang telah diekstrak dari tisu-tisu ditanam parafin dan diklonkan ke dalam vektor plasmid. Pada ketika pengklonan dan penurutan keseluruhan jarak gen ia telah menunjukkan rangka bacaan terbuka yang lengkap itu mengandungi 363 nukleotid dan menpunyai saiz yang sama dengan strain rujukan Cux-1 CAV. Gen yang didapati adalah 98 % homolgus (sama siri) kepada strain rujukkan dan juga tinggi pengekalan dengan gen

VP3 dari lain-lain strain CAV. Ciri-ciri fungsi gen VP3 juga disiasat dangan menggunakan sistem expressi eukaryotik, pcDNA 3.1/ Zeo + plasmid. Rekombinan plasmid pcDNA-VP3 yang telah ditulinkan telah digunakan terhadap sel mamalia transfeksi dan sel kanser melalui electroporasis. Apoptosis yang diaruhkan pada ketika expressi gen VP3 telah dikaji dengan mengenalpasti dan mengesan secara morfologi dan perubahan biokima yang disebabkan oleh apoptosis. Pada masa pemeriksaan microskop elektron transmisi, gambaran apoptosis yang tipikal telah dilihat termasuk pinggiran nuklear dan pembentukkan badan-badan apoptotik seawal-awal hari ke 2 selepas transfeksi. Perubahan morphologi terhasil disebabkan apoptosis kemudian ditentukan dengan pewarnaan secara biokimia apoptotik dan asai pemotongan DNA. Aktiviti-aktiviti VP3 yang berbeza kemudiannya diselidik dengan menggunakan teknik immunofluoresen. Menarik sekali, dalam sel-sel normal, protin VP3 yang dikesan hanya bersetempat dalam sitoplasm dan tidak dalam lain-lain bahagian sel-sel transfeksi. Sebaliknya, pada ketika transfeksi, protein VP3 terdapat dalam nukleusnya (Vero dan Fibroblas embrio tikus) dan sel-sel kanser payudara manusia. Kesan-kesan expresi protin VP3 dalam 3 jenis sel-sel ini juga telah di kenalpasti menerusi pemeriksaan analisa mikroskop konfokal dengan sel-sel yang diwarnakan menerusi propidium iodida dan acridine oren. Kesimpulannya, expressi protin VP3 sahaja mampu untuk mengaruh apoptosis dalam sel-sel yang transfeksi dan sel payudara manusia tetapi tidak berkesan ke-atas sel-sel normal. Bukti-bukti yang kuat menunjukkan. DNA yang dibentuk mengandungi gen VP3 dibawah kawalan pemaju sitomegalovirus manusia (hCMV) dan dalam bentuk sebagai vaksin DNA adalah mempunyai potensi baru dalam pengubatan anti-kanser.

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I certify that an Examination Committee met on the 5th October 2001 to conduct the final examination of Mohamed Ghrici on his Master of Science thesis entitled "The Expression of Chicken Anemia Virus VP3 Gene and Induction of Apoptosis in Transformed and Tumor Cells" in accordance with Universiti Pertanian Malaysia (Higher Degree) Act 1980 and Universiti Pertanian Malaysia (Higher Degree) Regulations 1981. The committee recommends that the candidate be awarded the relevant degree. Members of the Examination Committee are as follows:

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DECLARATION

I hereby declare that the thesis is based on my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at UPM or other institutions.

Mohamed Ghrici

Date: 8.11.2001

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LIST OF ABBREVIATIONS

ADP	Adenosine 5'-diphosphate
AO	Acridine orange
ATP	Adenosine triphosphate
ATV	Antibiotic trypsin versin
A_{260}	Absorbance at 260 nm
BCL-2	B cell leukemia / lymphoma 2
BCR-ABL	B Cell antigen receptor-Abelson murine leukemia
BGHpA	Bovine growth hormone polyadenylation
BHK	Baby hamster kidney
bp	base pair
BSA	Bovine serum albumin
BT	Bovine testis cells
°C	Degree celsius
$CaCl_2$	Calcium chloride
Ca/Mg	Calcium / Magnesium
cAMP	Cyclic Adenosine 3':5' monophosphate
CAT	Chloramphenicol acetyltransferase
CAV	Chicken anemia virus
ccdB	Control of cell death gene
CEF	Chicken embryo fibroblast
CNRS	Centre nationale dela recherche scientifique
CPE	Cytopathic effect
CrmA	Cytokine response modifier A
CsCl	Cesium chloride
Da	Dalton
ddH_20	Deionised distilled water
DMEM	Dulbecco's modified essential medium
Dnase	Deoxyribonuclease
d NTP	Deoxyribonucleotide
ds	Double-stranded
E.coli	Escherichia coli
EDTA	Ethylenediaminetetraacetic acid
e.g	For example
ELISA	Enzyme-linked immunosorbent assay
EM	Electron microscopy
EMEM	Eagle's minum essential medium
FBS	Fetal bovine serum
FITC	Fluorescein isothiocyanate
g	Gram
xg	Gravity
g/ml	Gram per ml
H_2O_2	Hydrogen peroxide
HBS	HEPES-buffered saline
Hbs	Hepatitis surface antigen (S)
HEPES	N-2-hydroxyethylpiperazine-N'-2 ethanesulfonate
hCMV	Human cytomegalovirus
hr	Hour
HSV/tk	Herpes simplex virus thymidine kinase

ICE	Interleukin-1β-converting enzyme
IgG	Immunoglobulin G
IIF	Indirect immunofluorescence
IPTG	Isopropylthiogalactopyranoside
kb	Kilobase
kDa	Kilodalton
L15 medium	Leibovitz's L15 medium
LB	Luria bertani
LM-PCR	Ligation-mediated PCR
LMP	Low melting point
Luc	Luciferase
M	Molar
Mab	Monoclonal antibody
mg	Milligram
mg/ml	Milligram per ml
min	Minute
ml	Milliliter
mM	Milimolar
MCF-7	Human mammary adenocarcinoma
MDA-MB	Human, caucasian, breast adenocarcinoma
MDCC	Marek's disease virus transformed chicken lymphocyte
MDV	Marek's disease virus
mRNA	Messenger ribonucleic acid
MW	Molecular weight
ug	Microgram
ul	Microliter
uM	Micromole
$NaHCO_3$	Sodium hydrogen carbonate
NaOH	Sodium hydroxide
ng	Nanogram
ng/ml	Nanogram per ml
nm	Nanometer
OD	Optical density
ORF	Open reading frame
pH	Hydrogen-ion activity
%	Percentage
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
PI	Propidium iodide
pmol/ul	Picomol per microliter
Rb	Retinoblastoma
RE	Restriction endonuclease
REF	Rat embryo fibroblast
RF	Replicative form
rpm	Revolution per minute
RPMI-1640	Roswell park memorial institute
SDS-PAGE	Sodium dodecyle sulphate-polyacrylamide gel electrophoreis
sec	Second
SPF	Specific pathogen free
ss	Single-stranded

TAE	Tris-acetate-EDTA buffer
TE	Tris-EDTA
TEM	Transmission electron microscope
TUNEL	TdT-mediated dUTP Nick-End Labeling
U	Unit
UV	Ultraviolet
V	Volt
vs	Versus
v/v	Volume per volume
w/v	Weight per volume
Xgal	5-bromo-4-chloro-3-indolyl-β-D-galactoside

CHAPTER 1

INTRODUCTION

The living organisms and the maintenance of their wellbeing and health are tribute to the equilibrium between cell division and apoptosis. The failure or dysregulation of one of these two fundamental mechanisms can be fatal or have serious pathological consequences (Singh *et al.,* 1994). Cellular death may occur either via apoptosis or necrosis. Necrosis is non-physiological or pathological type of cell death and it is uncontrolled in nature. It occurs following an extreme cell damage and is characterized by swelling and lysis of the cell causing the release of its content into the surrounding environment leading to inflammation (Trump *et al.,* 1991).

Apoptosis differ from necrosis particularly by two dominant markers: morphological marker characterizing the structural changes of apoptotic cell and the biochemical marker characterizing the cleavage of DNA into domain-sized fragments (Walker *et al.*, 1993). Apoptosis is a programmed cell death and is a genetically controlled cellular mechanism of cell death to eliminate unwanted cells (Earnshaw, 1995). Apoptosis is a widespread cellular mechanism involved in numerous processes such as the embryonic and neural development (Sanders and Wride, 1995), the regulation of the immune system (Williams, 1994), organogenesis and tissue homeostasis (Wyllie *et al.,* 1980). Some of the molecular and biochemical mechanisms of apoptosis have been elucidated and the research in this area continues to expand at an

extremely rapid rate following the development of new techniques and equipment (Hale *et al.,* 1996).

The interest in apoptosis rise from the findings that apoptosis was involved in many important diseases such as neurodegenerative disorders, AIDS and particularly cancer, which affect millions of people around the globe. The growing amount of data generated through a continuous investigations of the mechanisms of apoptosis has proved to be very valuable in understanding the basic cell biology which will path the way to the development of very sensitive diagnostic tools and the design of therapies for various diseases. The suppression or failure and/or excessive rate of apoptosis can trigger the development of tumors as well as render the tumor cells resistant to the current chemotherapy and radiotherapy (Williams, 1994; Kerr *et al.,* 1994; Thompson, 1995).

The elucidation of the molecular mechanisms underlying apoptosis cannot be achieved without developing appropriate methods for detecting and characterizing apoptosis. Classically, the detection and characterization of apoptosis was based on the examination of morphological changes at the cellular level by light-electron-microscopy in combination with vital fluorescent dyes (McGahon *et al.*, 1995). With the advancement of the technology new tools were designed to detect apoptosis at the molecular level, such as the use of Annexin V to monitor the loss of membrane phospholipid asymetry during apoptosis (Koopmans *et al.*, 1993) or assays to detect DNA fragmentation by agarose gel electrophoresis (Wyllie. 1980), or by in-situ nick-

end labeling (Gavrieli *et al.,* 1992). Assays to measure the disruption of the mitochondrial transmembrane potential and the measurement of DNA and RNA content by flow cytometry were developed later (Darzynkiewicz *et al.,* 1992). Flow cytometry and its various alternatives are the most powerful methods to detect and quantitate apoptotic cells (Darzynkiewicz *et al.,* 1997)

The important contribution of apoptosis research was made particularly in the field of oncology, where it was demonstrated that tumor was a consequence of failure or suppression of apoptosis, which also may render tumor cells more aggressive and resistant to chemotherapy and radiotherapy (Williams, 1991). Chemotherapeutic drugs and ionizing radiation, which are the only available cancer treatment, destroy the cancerous cells via apoptosis usually via the action of wild type p53 (Dive and Hikman, 1991; Fisher, 1994; Thompson, 1995). These current therapies become hopeless when a mutation strike the p53 gene and/or by the over-expression of bcl-2 (Lowe *et al.,* 1994). This problem may be solved by the use of a novel cancer therapy that would be independent to the present of prerequisite factors. VP3 protein is encoded by an avian virus (CAV) and has a length of 121 amino acids with an estimated size of 14 kDa (Noteborn *et al.,* 1994). The chicken anemia virus (CAV) was first isolated in Japan in 1979 (Yuasa *et al.,* 1979) and was characterized as a new viral pathogen belonging to *Circoviridae* family (Studdert *et al.,* 1993). In young chickens, CAV transiently cause severe anemia and immunodeficiency due to depletion of cortical thymocytes (Jeurissen *et al.,* 1989; Yuasa *et al.,* 1979). The depletion of thymocytes was demonstrated to